Chapter 30

In Vivo Analysis of Apoptosis in Embryonic Hippocampus

Ahmad Salimi and Jalal Pourahmad

Abstract

Apoptosis is fundamental in several morphogenetic processes and ultimately determines the mass, shape, and function of the various tissues and organs that form the animal body. This process is a gene-regulated process that plays fundamental roles in several normal and pathological conditions. Apoptosis is most often detected during embryonic development. Although the nervous tissue is traditionally regarded as being fundamentally constituted by postmitotic nonproliferating cells, analysis of cell proliferation and apoptosis in vivo has recently gained an increasing importance mainly during embryonic development because there is large evidence that drug-induced apoptosis is the most likely candidate for the behavioral deficits. These effects occur at therapeutically relevant blood levels, and require only a relatively brief exposure. We describe here a of techniques that are currently for the detection of apoptotic cells in the central nervous system (CNS) directly on tissue sections in postnatal mice.

Key words Apoptosis, Hippocampus, Drug, Mouse, Embryos

1 Introduction

The hippocampus is part of the cortex structure of the limbic forebrain, and its relation to cognitive function has become a popular research topic [1]. The hippocampus regulates visceral functions through sympathetic and parasympathetic nerves. In clinical practice, patients with cognitive effect often have abnormal levels of blood glucose and lipids, and hippocampal neuron apoptosis is the pathological characteristic and outcome of the hippocampus in these patients [2]. The immature brain is sensitive to widespread neuronal apoptosis secondary to a variety of insults including certain drugs [3, 4]. The observation that third-trimester gestational ethanol exposure can produce widespread neurobehavioral deficits and neuronal apoptosis led to the hypothesis that the adverse cognitive/behavioral effects of many drugs might be due to similar mechanism [5]. Widespread neuronal apoptosis occurs as a result of neonatal exposure to diazepam, phenobarbital, phenytoin, clonazepam valproate, or

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vigabatrin [6]. This chapter provides a detailed step-by-step method for the in vivo evaluation of apoptosis with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL).

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at appropriate temperature. Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Animal

1. Postnatal C56/BalbC mice. All experiments should be conducted in accordance with the national laws for the use of animals in research and approved by the local ethics committee.
2. Pentobarbital for anesthetizing animals (50 mg/kg IP).
3. 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4 at 4 °C for fixation of organ (*see* **Note 1**).
4. A surgical set for removing of brain from skull.

2.2 Preparation of Tissue

1. Ethanol at different concentrations for brain dehydration.
2. Butanol (10%, w/v).
3. Xylol.
4. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4 in dH_2O, pH 7.4 (with HCl).
5. 0.1 M citrate buffer.
6. 100% Paraplast.
7. 0.01% aqueous solution of poly-L-lysine.
8. Paraffin.
9. Microtome.
10. Microwave (*see* **Note 2**).

2.3 Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL)

1. Phosphate-buffered saline (PBS) (*see* Subheading 2.2).
2. 4% buffered formaldehyde: dilute high-quality formaldehyde (v/v) in PBS prior to use.
3. 20 μg/mL proteinase K. Stock solution may be stored at −20 °C for several months.
4. Ethanol (95%, 90%, 80%, and 70%) in Coplin jars.
5. 2% hydrogen peroxide. Prepare fresh from hydrogen peroxide reagent stock prior to use.
6. 2% BSA solution: 2% BSA (w/v) dissolved in PBS and passed through a 0.45 μm filter. Sterile stock solution may be stored at 4 °C for several weeks.

7. 2× saline–sodium citrate (SSC) buffer: 300 mM NaCl, 30 mM sodium citrate. Stock solution may be stored at room temperature for several months.
8. Terminal transferase (TdT) equilibration buffer: 2.5 mM Tris–HCl (pH 6.6), 0.2 M potassium cacodylate, 2.5 mM $CoCl_2$, and 0.25 mg/mL BSA. Prepare from stock solutions. Aliquots may be stored at −20 °C for several months.
9. TdT reaction buffer: TdT equilibration buffer containing 0.5 U/μL of TdT enzyme and 40 pmol/μL biotinylated-dUTP. Prepare fresh from stock solutions prior to use.
10. Vectastain ABC-peroxidase stock solution.
11. 3,3′-Diaminobenzidine (DAB) staining solution.
12. TdT staining buffer: 4× saline-sodium citrate (0.6 M NaCl, 60 mM sodium citrate), 2.5 μg/mL fluorescein isothiocyanate-conjugated avidin, 0.1% Triton X-100, and 1% BSA. Prepare fresh from stock solutions prior to use.
13. Hematoxylin counterstain.
14. 2 μg/mL Hoechst 33342 counterstain in PBS. Stock solution may be stored at 4 °C in the dark for several weeks.
15. Vectashield antifade mounting medium.

3 Methods

3.1 Tissue Preparation

1. Anesthetize postnatal mice with an intraperitoneal injection (800 μL) of pentobarbital and perfused intracardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4 at 4 °C.
2. Using sterile scissors or scalpel, open cranium of pup from back of the neck to the nose. Carefully remove the entire brain with forceps. Place the brain on sterile gauze. Using a sterile scalpel, remove the cerebellum and incise down the midline of the brain to separate it into two hemispheres (Fig. 1) (*see* **Note 3**).
3. Grasp a small section of meninges surrounding the hippocampus with sterile forceps and pull it gently away. In either case, the hippocampus will be more clearly visible after the meninges have been removed. The hippocampus is a curved structure that starts in the distal part of the hemisphere and bends ventrally.
4. Place the hippocampus into 4% PFA for 2 h at 4 °C.
5. After dehydration in graded ethanol baths (three times for 15 min in 50%, 70%, and 95% and three times for 1 h in 100% ethanol), hippocampus are incubated in 100% butanol for 12 h and then impregnated at 60 °C with 50% butanol–50% paraffin for 48 h before final incubation in 100% Paraplast for 24 h.
6. Cut paraffin sections of 10 μm thickness on a microtome.

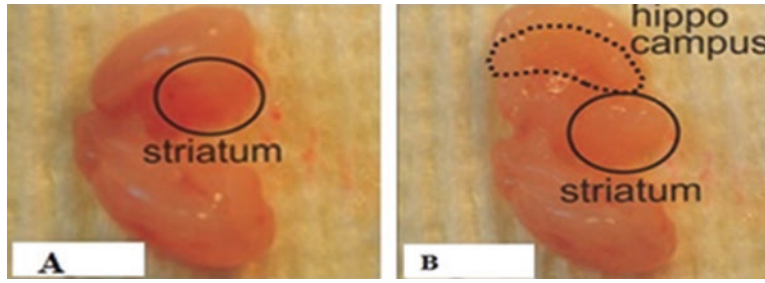

Fig. 1 (**a**) Dissection of the prenatal mouse brain. The first incision is down the midline of the brain separating it into two hemispheres. (**b**) Location of the hippocampus in the prenatal mouse brain. The striatum is moved aside to visualize the hippocampus and is noted by the curved "kidney bean" type structure in the distal region of each hemisphere

7. Treat the sections with xylol and butanol and rehydrated in ethanol baths (100%, 95%, 70%, 50%, and 0%) with a final step in 0.01 phosphate buffer saline (PBS).
8. To improve the efficiency of the TUNEL, incubate sections in 0.01 M citrate buffer pH 3 and heated three times for 5 min in the microwave at 350 W.

3.2 Colorimetric Staining for Light Microscopy

1. Fix tissue samples in 4% formaldehyde prepared in PBS for 24 h and embed in paraffin. Adhere 4–6 μm paraffin sections to glass slides pretreated with 0.01% aqueous solution of poly-L-lysine.
2. Deparaffinize sections by heating the slides for 30 min at 60 °C (or 10 min at 70 °C) followed by two 5-min incubations in a xylene bath at room temperature in Coplin jars.
3. Rehydrate the tissue samples by transferring the slides through a graded ethanol series: 2 × 3 min 96% ethanol, 1 × 3 min 90% ethanol, 1 × 3 min 80% ethanol, 1 × 3 min 70% ethanol, 1 × 3 min double-distilled water (DDW).
4. Carefully blot away excess water and pipet 20 μg/mL proteinase K solution to cover sections. Incubate for 15 min at room temperature.
5. Following proteinase K treatment, wash slides 3 × 5 min with DDW.
6. Inactivate endogenous peroxidases by covering sections with 2% hydrogen per-oxide for 5 min at room temperature. Wash slides 3 × 5 min with DDW.
7. Carefully blot away excess water and cover sections with TdT equilibration buffer for 10 min at room temperature.
8. Remove TdT equilibration buffer and cover sections with TdT reaction buffer. Incubate slides in a humidified chamber for 30 min at 37 °C (*see* **Note 4**).

9. Stop reaction by incubating slides 2 × 10 min in 2× SSC.
10. Rinse slides in PBS and block nonspecific binding by covering tissue sections with 2% BSA solution for 30–60 min at room temperature.
11. Wash slides 2 × 5 min in PBS and incubate in Vectastain ABC-peroxidase solution for 1 h at 37 °C.
12. Wash slides 2 × 5 min in PBS and stain with DAB staining solution at room temperature. Monitor color development until desired level of staining is achieved (typically 10–60 min). Stop the reaction by incubating slides in DDW.
13. Lightly counterstain tissue sections with Hematoxylin stain.
14. Cover tissue sections with coverslips using Aqua-Poly/Mount mounting medium.
15. Observe sections under light microscopy.

3.3 Fluorescent Staining

1. Repeat **steps 1–9** outlined in Subheading 3.2, omitting the hydrogen peroxide inactivation step.
2. Wash slides 2 × 5 min in PBS then cover tissue sections with TdT staining buffer. Incubate slides at room temperature for 30 min in the dark.
3. Wash slides 2 × 5 min in PBS.
4. Lightly counterstain sections with Hematoxylin, Hoechst 33342, or another appropriate counterstain.
5. Wash slides with PBS, air-dry, and attach coverslips using Vectashield antifade mounting medium.
6. Examine tissue sections by fluorescence or confocal microscopy.

4 Notes

1. Make fresh 4% paraformaldehyde each time. Preparation should be carried out inside a fume hood. Store it at 4 °C for up to 1 week.
2. The use of the microwave at 350 W for heating is to improve the efficiency of the Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL).
3. Make sure that the most of blood is washed away (blood inhibits fixation). Heads of older embryos can be cut by half sagittally at midline using razor blade. Each half can be embedded separately.
4. In order to conserve reagents a reduced volume of TdT buffer may be carefully covered with a glass coverslip during the incubation. Take care to avoid trapping air bubbles which may lead to staining artifacts.

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